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An aromatic cap seals the substrate binding site in an ECF-type S subunit for riboflavin

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Abstract

ECF transporters are a family of active membrane transporters for essential micronutrients, such as vitamins and trace metals. Found exclusively in archaea and bacteria, these transporters are composed of four subunits: an integral membrane substrate-binding subunit (EcfS), a transmembrane coupling subunit (EcfT), and two ATP-binding cassette ATPases (EcfA and EcfB). We have characterized the structural basis of substrate binding by the EcfS subunit for riboflavin from *T. maritima*, TmRibU. TmRibU binds riboflavin with high affinity, and the protein-substrate complex is exceptionally stable in solution. The crystal structure of riboflavin-bound TmRibU reveals an electronegative binding pocket at the extracellular surface in which the substrate is completely buried. Analysis of the intermolecular contacts indicates that nearly every available substrate hydrogen bond is satisfied. A conserved aromatic residue at the extracellular end of TM5, Tyr130, caps the binding site to generate a substrate-bound occluded state, and nonconservative mutation of Tyr130 reduces the stability of this conformation. Using a novel fluorescence binding assay, we find that an aromatic residue at this position is essential for high affinity substrate binding. Comparison with other S subunit structures suggests that TM5 and Loop5-6 contain a dynamic conserved motif that plays a key role in gating substrate entry and release by S subunits of ECF transporters.

Graphical Abstract

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ACCESSION NUMBERS

The atomic coordinates and structure factors for the monoclinic, hexagonal and orthorhombic TmRibU crystal structures have been deposited in the Protein Data Bank as 5KBW, 5KC0, and 5KC4, respectively.

AUTHOR CONTRIBUTIONS

N.K.K. designed and performed the experiments with J.S. N.K.K. and D.N.W. analyzed the data and wrote the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental information includes 8 figures and 1 table and can be found with this article online at...

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Keywords

Membrane transport; X-ray crystallography; vitamins; lipidic cubic phase; thermal stability

Introduction

Energy coupling factor (ECF) transporters are a family of primary active membrane transporters for the uptake of essential micronutrients, such as vitamins and trace metals [1]. Found exclusively in archaea and bacteria, including the human pathogens *Listeria*, *Streptococcus*, and *Staphylococcus*, ECF transporters may be the only means of vitamin acquisition in these organisms [1–5]. ECF transporters are composed of four subunits: a high affinity integral membrane substrate-binding subunit (EcfS), a transmembrane coupling subunit (EcfT), and two homologous ATP-binding cassette (ABC)-type ATPases (EcfA and EcfA') that use ATP to drive substrate uptake [6]. As a result, ECF transporters can be considered a unique sub-family of the ABC transporter superfamily of ATP-dependent pumps [7, 8].

ECF transporters can be divided into two groups based on the relative chromosomal location of the ECF and S subunit genes [1]. Both groups utilize an energizing module composed of the EcfA, A', and T subunits, also known as the energy coupling factor for which the transporter family is named [9]. In the group I transporters, the four genes are present in the same operon, and these proteins form a dedicated complex for uptake of a single substrate as typified by the ECF transporter for biotin from *Rhodobacter capsulatus* [6]. In contrast, multiple S subunits for distinct substrates share a common energizing module in the group II members [1], and the S subunit genes are encoded separately from the ECF. In this way, the substrate specificity of group II ECF transporters is conferred via interaction with a particular S subunit.

X-ray crystal structures of S subunits for riboflavin (RibU) [10], thiamine (ThiT) [11], biotin (BioY) [12], Co^{2+} (CbiM) [13], and folate (FolT) [14, 15] have been determined in isolation from the ECF module. In addition, crystal structures of the entire EcfAA'T-S transporter complexes for folate [15, 16], hydroxymethyl pyrimidine [17], and pantothenate [18] have been solved. Despite the limited sequence identity between S subunits for chemically diverse substrates, these structures revealed a conserved core of six transmembrane (TM) helices with a high affinity substrate binding site located at the extracellular surface [19]. The six helices can be considered as two functional subdomains, whereby TM1-3 interact with the EcfT subunit while residues from TM4-6 are directly responsible for substrate binding [12]. In the S subunit structures, the bound substrate is enclosed by a long loop on the extracellular side that connects TM1 and TM2, called Loop1-2. Upon substrate binding to ThiT in solution, Loop1-2 undergoes a large conformational change from an open to a closed state in order to sequester thiamine in the binding site [20]. Comparison of apo and

folate-bound FolT structures also found that Loop1-2 changes conformation during substrate binding [14].

While the available data is consistent with an important role for Loop1-2, it is unknown whether other structural elements play a dynamic role in substrate binding by S subunits [19]. Moreover, the first RibU crystal structure, from the *S. aureus* homolog (SaRibU), was determined to a modest resolution of 3.6 Å [10], which precluded determination of the detailed protein-substrate interactions. To address these questions, we have characterized the role of TM5 and Loop5-6 in substrate recognition and release by a RibU EcfS subunit using a combination of structural and biochemical approaches.

RESULTS

Characterization of the riboflavin binding EcfS subunit from *T. maritima*, TmRibU

We expressed and purified the EcfS subunit for riboflavin from *Thermotoga maritima*, TmRibU. In the detergent nonyl glucoside (NG), TmRibU eluted from size exclusion chromatography (SEC) as a single, sharp symmetrical peak (Figure 1a). Notably, TmRibU co-purified with the riboflavin transport substrate, as evidenced by the yellow color of the purified protein and the visual absorbance spectrum with peaks at 380 and 462 nm (Fig. 1b) [10, 21]. Indeed, the protein-substrate complex was so stable in solution that it failed to denature during SDS-PAGE, as detected by the long wavelength UV fluorescence of the purified protein bands (Fig. 1a inset). The co-purification of transport substrate and the extraordinary stability of the TmRibU-riboflavin complex are consistent with the nanomolar binding affinity and slow off rate reported for the *L. lactis* RibU homolog [21]. Upon co-expression with the EcfA, EcfA', and EcfT subunits, in *E. coli* His-tagged TmRibU pulled down all three components of the *T. maritima* ECF module (Fig. 1c). These observations indicated that our protein preparation was active and highly stable.

As there has been some controversy over whether EcfS subunits function as monomers or dimers [10, 12, 22–25], we determined the oligomeric state of riboflavin-bound TmRibU in solution. Multi-angle laser light scattering analysis (MALLS) [26] of riboflavin-bound TmRibU in detergent solution indicated a molecular mass of 21.6 ± 0.7 kDa for the substrate-bound protein component of the protein-detergent complex (Fig. 1d). As the expected molecular weight of the TmRibU construct used in this study plus bound riboflavin is 21.1 kDa, these observations are in good agreement with the purification of a stable, TmRibU-riboflavin complex. Consequently, these results indicate that the TmRibU S subunit is a monomer in isolation from the EcfAA'T energizing module.

Crystal structure of riboflavin-bound TmRibU

In order to determine the structural basis of high affinity substrate binding and the conformational changes required for vitamin entry and release from an EcfS subunit, we determined the crystal structure of riboflavin-bound TmRibU. The best diffracting crystals required truncation of the last 10 carboxy-terminal residues of TmRibU, which were predicted to be disordered *in silico* [27]. Importantly, this variant of TmRibU, (TmRibU_{Ct}) still assembled with the other ECF subunits to form a complex (Fig. 1c). We obtained three

unique crystal forms of riboflavin-bound, TmRibU Ct: a monoclinic crystal grown from lipidic cubic phase [28] with monoolein as the host lipid, and hexagonal and orthorhombic crystals grown by vapor diffusion in the detergent NG (Table 1). While each crystal was a distinct space group, the TmRibU structures are essentially identical, with root mean squared deviations of ~ 0.6 Å for the five molecules in the asymmetric units of the three crystal forms. The monoclinic crystal form was refined at a resolution of 2.6 Å with R_{work} and R_{free} of 21.1 and 25.5%, respectively, and we used this structure for our analyses (Fig. S1).

The TmRibU structure contains six transmembrane (TM) α -helices with both amino and carboxy-termini in the cytoplasm. The helices are arranged in two layers of three roughly parallel helices formed by TM1-2-6 and 3-4-5 (Fig. 2a). A single riboflavin substrate molecule is bound in an electronegative pocket formed by TMs-4-6 at the extracellular surface (Fig. 2b). The substrate binding site is enclosed by the long loop connecting TM1 and 2 (Loop1-2) as observed in structures of S subunits for other vitamins: SaRibU [10], the thiamine-binding S subunit, ThiT [11], the S subunit for biotin, BioY [12], and folate-bound FolT [14, 15]. Within the binding pocket, the riboflavin substrate is almost completely buried (Fig. 2b), with $\sim 98\%$ of the solvent accessible surface area engaged at the protein interface.

Structural basis of high affinity flavin binding

The higher resolution of our TmRibU crystals compared to the SaRibU structure [10] allowed us to evaluate the detailed molecular interactions in the riboflavin binding site. Riboflavin is composed of a tricyclic isoalloxazine ring system linked to an open chain ribityl tail. Upon binding to TmRibU, the hydrophobic portion of the ring is enclosed in a cavity formed by Val75, Gly76 and Met79 from TM4, Phe33 from Loop1-2, and Ile126 and Tyr130 from TM5 (Fig. 2c and d). The two polar rings of the tricyclic system are recognized by residues that are highly conserved among RibU homologs: Asn123 of TM5, Asn149 and Lys152 of TM6 (Fig. 2e, Fig. S2). The hydroxyl groups of the ribityl tail form hydrogen bonds (H-bonds) with Gly76 and Asn80 of TM4, Glu24 of TM1, and the backbone of Lys35 in Loop1-2. As a result, seven of the eight available H-bonds on the riboflavin substrate are satisfied via interactions with conserved residues from TM4-6 and Loop1-2 of TmRibU (Fig. 2f).

Interestingly, residues at the equivalent positions to Lys35, Asn149 and Lys152 of TmRibU are utilized for substrate recognition by BioY and FolT (Fig. S3). Similarly, the thiamine-binding His125 of ThiT is analogous to Asn123 of TmRibU (Fig. S3c), while residues from the same region of TM4 in all three structures form H-bonds with the transport substrate (Fig. S3e). Thus, in all of available structures of substrate-bound S subunits, most of the available transport substrate H-bonds are satisfied via interaction with specific amino acid positions in EcfS. This structural conservation highlights the amazing ability of S subunits to recognize chemically diverse substrates by minor alterations of a common protein scaffold.

Notably, the only available functionality on riboflavin that does not H-bond to TmRibU is the terminal 5' hydroxyl group in the ribityl tail (Fig. 2f). In cells, this moiety is phosphorylated to generate the redox cofactor flavin mononucleotide, or FMN. In this way,

the TmRibU binding site is tailored to accept either riboflavin or FMN. Indeed, studies on the substrate specificity of RibU indicate high affinity binding of riboflavin, FMN, and the antibiotic roseoflavin [21, 29, 30].

Previous biochemical work has investigated the riboflavin binding site of RibU. Studies of substrate binding by RibU from *L. lactis* concluded that residues Trp79 and Trp97 were not involved in substrate binding. However, mutation of Trp68 to Tyr reduced the K_d for riboflavin binding by ~100 fold [21]. In TmRibU, the equivalent residues are Pro74, Gly92, and Phe66, respectively (Fig. S4). Consistently, Pro74 and Gly92 are located at opposite ends of TM4 and do not contribute to the riboflavin binding pocket. In contrast, Phe66 resides at the extracellular end of TM3 and is ~4 Å away from the ribityl tail of bound riboflavin. Thus, the riboflavin binding site in the TmRibU crystal structures is in good agreement with the available biochemical data.

A conserved motif in TM5 caps the substrate binding site

Comparison of the TmRibU and SaRibU [10] crystal structures reveals conformational changes that may gate substrate entry and release from the binding site at the extracellular surface of EcfS subunits. These two proteins share 31% sequence identity, and the crystal structures display a 1.8 Å r.m.s.d for 165 residues upon C α superposition. In both structures, a conserved aromatic residue from Loop1-2 directly contacts the hydrophobic portion of the flavin ring. Specifically, both Phe33 of TmRibU and Tyr41 of SaRibU make an “edge-to-face” aromatic interaction [31] with the bound substrate (Fig. 3a). As a result, the conformation of Loop1-2, which undergoes a structural transition upon substrate binding [14, 20], is largely conserved between the two structures.

In contrast, TM5 and the intervening loop between TM5 and TM6, Loop5-6, adopt a radically different conformation in the TmRibU and SaRibU structures. In TmRibU, TM5 is straight, extends ~7 Å above the membrane surface and terminates in a Pro-Leu-Tyr (PLY) sequence motif that is highly conserved among RibU homologs (Fig. 3b). The tyrosine of this PLY motif, Tyr130 in TmRibU, sits on top of the substrate binding site and “caps” the hydrophobic portion of the flavin ring at the extracellular surface to seal the substrate in the binding site (Fig. 3a). In the SaRibU structure, the TM5 helix breaks upon reaching the membrane surface, and the extracellular segment swings out of the binding site. In addition, Loop5-6 was disordered in the crystal and is absent from the structure. These conformational changes cause Tyr139 from the PLY motif to retract ~6 Å from the binding site and partially expose the substrate to the extracellular environment (Fig. 3a). Analysis of the crystal packing in the TmRibU structures reveals that the PLY motif does not participate in any interactions in the hexagonal and monoclinic crystals (Fig. S5a). Similarly, the PLY motif of molecule B of SaRibU does not make any crystal packing contacts (Fig. S5b), indicating that the differences between the structures are not due to the crystallization environment. As a result, these observations suggest that the extracellular end of TM5 and Loop5-6 undergo a structural transition during riboflavin binding to RibU: an open state in which the PLY motif has swung out of the binding site, as observed in SaRibU [10], and an occluded conformation captured in the TmRibU crystal structures (Fig. 3a).

Additional evidence suggests that the PLY motif plays a dynamic, functionally important role in gating access to the substrate binding site of RibU. The observed B-factors in TM5 and Loop5-6 of SaRibU and the orthorhombic crystal form of TmRibU are amongst the highest in the structures, suggesting that this region is indeed dynamic (Fig. S6). Consistently, a molecular dynamics (MD) study on substrate binding to SaRibU found that TM5 and Loop5-6 undergo an induced fit motion upon substrate binding [32]. Therefore, as TM5 contains highly conserved residues that contact the bound substrate in the TmRibU structure, we propose that the PLY motif together with Loop1-2 control substrate entry and release from the binding site at the extracellular surface of RibU.

Tyr130 of the PLY motif stabilizes the substrate-bound occluded conformation

The RibU crystal structures and the MD simulations suggest that Tyr130 of TM5 undergoes a conformational change to stabilize the substrate-bound occluded state of the EcfS subunit. Accordingly, mutations of Tyr130 that increase the off rate for riboflavin would be expected to decrease the observed stability of the substrate-bound complex. As substrate binding can confer an increase in the thermal stability to proteins [33–36], we determined the melting temperature (T_m) of TmRibU and several mutants of Tyr130 in solution. In the relatively harsh detergent NG, the TmRibU-riboflavin complex displayed an apparent melting temperature of $87.1 \pm 0.7^\circ\text{C}$ (Fig. 3c), indicating an exceptionally high stability for the protein-substrate complex. As expected, mutation of Asn123 to alanine, which removes two protein-substrate H-bonds from the binding site, substantially decreased the T_m of the protein in solution. Mutation of Tyr130 to phenylalanine, which preserves the aromatic lid of the riboflavin binding pocket, yielded a T_m of $83.6 \pm 1.0^\circ\text{C}$. Indeed, in a broad alignment of RibU homologs, a tryptophan or phenylalanine is also found in place of tyrosine in the PLY motif, indicating that an aromatic residue is required at this position (Fig. 3b). In contrast to the Tyr130Phe mutation, the T_m of the Tyr130Gly mutant was reduced by $\sim 9^\circ\text{C}$ relative to the WT protein to $77.9 \pm 1.1^\circ\text{C}$ (Fig. 3c). As a result, mutation of Tyr130 to glycine, which removes the aromatic capping interaction and should facilitate substrate release, substantially reduces the stability of the substrate-bound, occluded state of TmRibU. Importantly, these mutants display an identical SEC profile to the WT protein and are bound to riboflavin in a 1:1 ratio (see Methods), indicating that the reduced T_m 's we observe do not reflect aggregation but instead the loss of key protein-substrate interactions.

Tyr130 of the PLY motif is essential for high affinity substrate binding

Our data suggest that the conserved tyrosine of the PLY motif of TmRibU plays an important role in substrate binding. To test this hypothesis, we sought to determine the substrate binding affinity to TmRibU and several mutants of Tyr130. For such experiments, a substrate-free protein preparation is required. However, due to the high affinity for substrate, our attempts to produce wild type TmRibU that was devoid of riboflavin or to dissociate the bound flavin after purification were unsuccessful (data not shown).

In order to circumvent these technical difficulties, we developed a novel experimental strategy to detect riboflavin binding by RibU. We took advantage of the observations that while isolated S subunits co-purify with their respective transport substrate [10–12, 15, 21, 37] complete ECF transporters do not [15–18, 25, 37, 38]. We previously demonstrated that

the homologous ECF riboflavin transporter from *Listeria monocytogenes*, LmECF-RibU, captures the riboflavin transport substrate by a “release and catch” mechanism [37]. In this system, ATP binding drives a conformational change that dissociates the EcfS subunit from the EcfAA'T ECF module. Upon release from the ECF module, the isolated RibU EcfS subunit tightly binds the transport substrate, which we can detect by the riboflavin fluorescence of the substrate-bound protein during size exclusion chromatography (FSEC) [39].

In order to evaluate the role of the conserved tyrosine in in transport substrate binding by an EcfS subunit, we determined the riboflavin binding affinity of LmRibU after ATP-dependent release from the ECF module. In this assay, WT LmRibU bound riboflavin with a K_d of 526 ± 74 nM (Fig. 4a, d). Similarly, the LmRibU Tyr136Phe mutant, which preserves the aromatic capping interaction with the substrate, bound riboflavin with a K_d of 566 ± 97 nM (Fig 4b, d). In contrast, the Tyr136Gly mutation completely abolished the high affinity binding of riboflavin (Fig. 4c, d). These findings are consistent with the key role of the the PLY motif in riboflavin binding and strongly agree with the TmRibU crystal structures (Fig. 2) and the thermal stability data (Fig. 3c). Importantly, these Tyr136 mutants of LmRibU did not impact the interaction with the ECF module (Fig. S7).

Previous studies have determined that EcfS subunits exhibit high binding affinity for the transport substrate in isolation from the ECF module (Table S1) [12, 14, 21, 22, 40]. Indeed, purified RibU from *L. lactis* (LIRibU) binds riboflavin with a K_d of 0.6 nM [21]. Notably, the apparent binding affinity of LmRibU for riboflavin is roughly 500-fold lower than LIRibU. To gain insight into the differences in the substrate binding affinity between the two homologs, we constructed homology models of LmRibU and LIRibU and compared the binding sites to the TmRibU structure (Fig. S8). This comparison suggests that the reduced binding affinity of LmRibU arises from several sequence variations compared to the other RibU homologs. First, the structural equivalent of Asn149 from TmRibU (Asn177 in LIRibU), which makes two H-bonds with riboflavin in the crystal structures (Fig. 2f) [10], corresponds to Ser160 in LmRibU (Fig. S8c). Therefore, this variation removes at least one H-bond from the substrate binding site. Second, the cavity responsible for binding the hydrophobic portion of the flavin ring in the TmRibU crystal structures (Fig. 2c and d) is composed of Ile80, Gly81 and Met84 from TM4, Phe35 from Loop1-2, and Ala132 and Tyr136 from TM5 in LIRibU (Fig S8b). In contrast, the analogous cavity in LmRibU consists of Ile80, Gly81 and Ser84 from TM4, Phe35 from Loop1-2, and Leu132 and Tyr136 from TM5 (Fig. S8c). As a result, the reduced hydrophobicity of this cavity due to the presence of Ser84 probably plays a role in the lower affinity of LmRibU for the riboflavin transport substrate. Moreover, as the three proteins share ~30% sequence identity, it is likely that a combination of subtle differences also contributes to the reduced riboflavin binding affinity of LmRibU relative to LIRibU.

The “aromatic cap” of TM5 is conserved in S subunits for other vitamins

Given the key role of Tyr130 in stabilizing the riboflavin-bound occluded state of TmRibU (Fig. 5a), we examined whether S subunits for other vitamins utilize a similar mechanism to cap the substrate binding site. Comparison of the available structures suggests that many

other S subunits also utilize an aromatic lid at the extracellular end of TM5 to sequester the transport substrate. In the thiamine-bound ThiT structure [11], Trp133 forms a face-to-face aromatic interaction with the substrate, sealing the binding site from the extracellular space (Fig. 5b). Like Tyr130 in RibU, this tryptophan is highly conserved among ThiT homologs [22] and these residues are located at approximately the same position in the two structures (Fig. 5c). Consistently, mutation of Trp133 to alanine in ThiT reduced the thiamine binding affinity over 1000-fold, but the phenylalanine mutant bound substrate near WT levels [22].

In addition to ThiT, the S subunits for folate (FolT), hydroxymethyl pyrimidine (HmpT), and pantothenate (PanT), also possess a conserved aromatic residue at the end of TM5. In the folate-bound FolT structure, Tyr129 sits directly over substrate binding site (Fig. 5d), and mutation of this tyrosine to alanine reduced the folate binding affinity by ~20 fold [14]. Similarly, both Phe128 in HmpT (Fig 5e), which is a tyrosine in some homologs [17], and Phe142 of PanT (Fig. 5f) [18], reside at the extracellular surface directly above the substrate binding site. This structural conservation between S subunits for different vitamins suggests a common mechanism for sealing the substrate from the extracellular space.

DISCUSSION

We have characterized the structural basis of high affinity substrate binding and release by TmRibU, the riboflavin binding EcfS subunit from the *T. maritima* group II ECF transporter for riboflavin. TmRibU is a monomer in isolation from the ECF module and binds the transport substrate with high affinity in a 1:1 complex (Fig. 1), as has been observed for other RibU homologs [10, 21]. The TmRibU-riboflavin complex is exceptionally thermostable, with an apparent T_m of ~87°C even in the relatively “harsh” detergent NG. These data indicate that TmRibU is one of the most stable integral membrane protein domains that have been measured to date. By comparison, other bacterial integral membrane proteins studied by this method include the hydrosulfide channel from *C. difficile*, the dicarboxylate transporter from *V. cholera*, and the YajR major facilitator from *E. coli*. These mesophilic proteins displayed melting temperatures of ~55, 44, and 64°C, respectively [33, 41]. In addition, the very stable, water soluble green fluorescent protein displayed a T_m of ~76°C when measured by a similar method [34, 42]. As membrane protein stability is negatively correlated with the chain length of the detergent used for purification [43], it is likely that TmRibU would display an even greater T_m in a “mild” detergent such as DDM.

The extreme thermostability of riboflavin-bound TmRibU probably arises from two factors. First, the source organism, *T. maritima*, is a hyperthermophile with a preferred growth temperature of 80°C [44], and proteins from these organisms have evolved resistance to thermal denaturation [45]. Biochemical studies have revealed that proteins from thermophiles display reduced dynamics compared to their mesophilic counterparts [46, 47]. Such reduced dynamics in TmRibU (Fig. S6) may have allowed us to capture the closed substrate-bound conformation. The second factor is the high substrate binding affinity, which arises from H-bonds between most of the available functionalities of the transport substrate and residues from TM4-6 and Loop1-2 (Fig 2e-f). Overall, 17 different residues, or ~10% of the total, make contact with riboflavin in the TmRibU crystal structures (Fig. S2). These multiple contacts between the transport substrate and the center of the protein

probably counteract the unfolding at elevated temperatures. Thus, the apo form of TmRibU, as well as S subunits for other vitamins, is probably significantly less stable relative to the substrate-bound state. Indeed, mutation of a single key substrate binding residue of TmRibU, Asn123, to alanine reduced the T_m by $\sim 23^\circ\text{C}$ (Fig. 3c). Similarly, BioY from *L. lactis* could not be purified in harsh detergents in the absence of the biotin transport substrate [12].

As TmRibU is the only EcfS protein to be studied by this method, we can not rule out that S subunits from mesophiles are also exceptionally resistant to thermal denaturation. Systematic investigations into the structural underpinnings of thermal stability in soluble proteins have found that thermophiles tend to possess fewer cavities and shorter loops, in addition to more salt bridges and optimized packing of the hydrophobic core [48–50]. For α -helical integral membrane proteins, closer packing of TM helices [51], reduced cavity volume [52], and increased hydrophobicity of the TM regions [53] are enriched in the limited number of membrane protein structures from thermophiles. However, there are relatively few structures of the same membrane protein homolog from both mesophilic and thermophilic sources. Further studies, such as determination of the structures and thermal stabilities of other RibU homologs from bacteria and archaea, could provide insight into the physical properties underlying thermostability in membrane proteins.

In this work we show that the loop connecting TM5 and 6 of TmRibU, Loop5-6, contains a functionally important aromatic residue that seals the substrate binding site in many S subunits. Several lines of evidence indicate an essential role for this structure in substrate binding. In the crystal structures of TmRibU, ThiT [11], and FoIT [14], the conserved aromatic residue in TM5 residue directly contacts the substrate to form the substrate-bound occluded state (Fig. 5a–d). Mutation of Tyr130 in TM5 of TmRibU to glycine significantly reduces the stability of this conformation (Fig. 3c), and the analogous mutation in LmRibU abolishes high affinity substrate binding (Fig 4). Similarly, mutation of the structurally homologous Trp133 to alanine in ThiT reduces the thiamine binding affinity by three orders of magnitude [22]. Thus, we conclude that TM5 and Loop5-6 of EcfS subunits, along with Loop1-2, play a key role in substrate binding and stabilization of the occluded state.

In addition to its role in substrate binding, the available data suggests that TM5 and Loop5-6 of RibU are dynamic and undergo a conformational change between the apo and substrate-bound states. In the SaRibU structure [10], the extracellular end of TM5 has swung out of the binding site and the conserved Tyr of the PLY motif does not contact the substrate (Fig. 3a). The B-factors of this region are among the highest in this crystal structure (Fig. S6f) and several of the residues that connect to TM6 were disordered. In MD simulations of SaRibU, this region was also found to undergo an open to closed transition upon riboflavin binding [32]. Notably, this “capping” of the substrate binding site in the simulation strongly depended on hydrophobic interactions, which is consistent with the hydrophobic pocket formed by Val75, Gly76 and Met79 from TM4, Phe33 from Loop1-2, and Ile126 and Tyr130 from TM5 in the TmRibU crystal structure (Fig. 2c and d). Similarly, MD simulations of pyridoxamine binding to HmpT suggested that Loop1-2 and Loop5-6 play a key role in gating substrate entry into the binding site [54]. Furthermore, in spectroscopic studies of substrate binding to ThiT, a probe located in Loop5-6, was found to be less mobile upon

substrate binding [20]. Taken together, these data indicate that the loops connecting TM helices 1–2 and 5–6 gate substrate entry and release from the binding site at the extracellular surface in many S subunits of ECF transporters.

Our findings have implications for the transmembrane transport mechanism of group II ECF transporters. Several studies indicated that S subunits for different vitamins compete for transport by the ECF module and such competition is enhanced in the presence of the substrate [9, 37, 55]. These observations suggest that the apo and substrate-bound conformations of EcfS have different affinities for the ECF module. As both Loop1-2 and the Loop5-6 undergo a conformational change to bury the substrate in the binding pocket in RibU, this substrate-bound, occluded state could present a unique binding surface for the ECF module. Such a mechanism would maximize the efficiency of transport through the selective recognition of substrate-loaded EcfS by the ECF module. Moreover, through the regulated expression of specific S subunits [29, 30, 40, 56], cells could readily adapt to differences in substrate availability while utilizing a single transporter platform.

EXPERIMENTAL PROCEDURES

Expression and purification of TmRibU

The gene for TmRibU was amplified from *T. maritima* MSB8 genomic DNA (ATCC 43589), and cloned into a modified pBAD vector (Invitrogen) with a carboxy-terminal myc-decahistidine tag linked by a thrombin recognition site. This construct was transformed into *E. coli* BL21(DE3), and protein expression was performed in LB media at 37 °C. Cells were resuspended in 50 mM Tris, pH 7.5, 500 mM NaCl, 20% glycerol and 10 mM imidazole. After cell breakage in a cell disruptor, unlysed cells were removed by centrifugation and the supernatant was treated with 1% dodecyl-maltoside (DDM) for membrane protein solubilization. His-tagged TmRibU was bound to Talon resin (Clontech) in DDM and exchanged into SEC buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.4% NG) by thrombin cleavage from washed beads. TmRibU was further purified by gel filtration on a Superdex 200 column equilibrated in SEC buffer. A construct that truncates the 10 carboxy-terminal residues of TmRibU, TmRibU_{Ct}, was used for all crystallization experiments.

Crystallization and structure determination of TmRibU

For crystallization in NG, SEC peak fractions of TmRibU_{Ct} were pooled and concentrated to ~8mg/ml. The hexagonal crystals of TmRibU were grown by vapor diffusion from drops consisting of 1 µL protein and 1 µL 100 mM sodium acetate, pH 4.5, 28% PEG400, and 50 mM magnesium acetate at 4 °C. The orthorhombic crystals were grown from drops consisting of 1 µL protein and 1 µL 100 mM Tris, pH 8.5, 30% PEG400, 100 mM NaCl, 0.5% NG, and 1% hexanediol at 16 °C. For crystallization in the lipidic cubic phase, SEC peak fractions were pooled, concentrated to ~11mg/ml, and filtered with 0.22 µm spin filters. Subsequently, the concentrated protein stock was mixed with liquid monolein at a 40% w/v ratio by the syringe mixing technique [28]. Monoclinic crystals were grown on glass sandwich plates (Molecular Dimensions) consisting of 80 nL TmRibU_{Ct} in LCP and 1 µL 100 mM sodium acetate, pH 5.0, 30% PEG500 DME, and 100 mM NaCl at 20 °C.

Diffraction data were collected at beamlines X25 and X29 of the NSLS, and beamline 8.2.1 of the ALS, and all data were processed with HKL2000 [57]. The P6₅22 TmRibU crystal structure was determined by molecular replacement in Phaser [58] with the polyaniline SaRibU structure [10] as the search model. After sequence assignment and rebuilding, the P2₁2₁2₁ structure and the P2₁ structures were solved with this refined model. All structures were built with Coot [59] and refined with PHENIX [60]. The final model of the monoclinic and hexagonal crystal forms contains residues 2–172 and 2–166 of TmRibU, respectively. The final model of the orthorhombic crystal form contains residues 2–166 of TmRibU, except for 70–73 of molecule B, which were not apparent in the electron density. Structure figures were prepared with Pymol [61].

Expression and purification of TmECF-RibU complexes

The EcfA and A' genes were cloned into pACYC-Duet (EMD4Biosciences) for co-expression. Wild type and the Ct mutant of TmRibU were cloned into a modified pET15 vector (EMD4Biosciences) fused to an amino-terminal decahistidine tag. For co-expression of the TmRibU and EcfT subunits, these genes were linked by a ribosome binding site (underlined) with the following sequence:
5'ATAATTTTGTTTAACTTTAAGAAGGAGATATACC3'. Protein expression and purification was performed as described in [25]. The purified TmECF-RibU complexes were detected by SDS-PAGE.

SEC-MALLS Analysis of TmRibU

Purified TmRibU at a concentration of ~4 mg/ml was injected onto a Shodex 803 column equilibrated in 50 mM Tris, pH 7.5, 200 mM Na₂SO₄, 2 mM NaN₃ and 0.05% DDM. This column was run on an HPLC (Waters, MA) attached to a MiniDawn Treos multi-angle light scattering detector and an Optilab Rex refractive index detector (Wyatt Technologies, Santa Barbara, CA). UV absorbance, light scattering, and refractive index data were analyzed according to the method described in [26]. For the TmRibU-riboflavin complex, an extinction coefficient at 280 nm of 1.22 was used, which takes the 280 nm of absorbance of the bound flavin into account. This was calculated as $((\# \text{ Trp} * 5500) + (\# \text{ Tyr} * 1490) + (\# \text{ B2} * 18167)) / (\text{TmRibU molecular weight} + \text{B2 molecular weight}) = ((0 * 5500) + (5 * 1490) + (1 * 18167)) / 21071$.

T_m determination of TmRibU

The apparent T_m of TmRibU was determined by the methodology described in [33]. Briefly, TmRibU at a concentration of 1 mg/ml was incubated in a thermal cycler for 10 min at the indicated temperatures and then kept on ice. After centrifugation, 50 µl of each sample was injected on a Shodex KW804 analytical SEC column (Thomson, Clear Brook, VA) equilibrated with 50 mM Tris, pH 7.5, 200 mM Na₂SO₄, 3 mM NaN₃ and 0.05 % DDM on an HPLC (Waters, Milford, MA). SEC peak heights were determined with the Empower software (Waters). The data in Figure 3c represents the mean and standard deviation of two independent replicates for each sample. All curve fitting and graphs were performed with Prism 6, GraphPad Software, Inc. The Asn123Ala, Tyr130Phe, and Tyr130Gly, and N123A TmRibU mutants are bound to riboflavin in a 1:1 ratio as indicated by the ratio of the 440 to 280 nm absorbance of the purified protein.

Expression and purification of LmECF-RibU complexes

The LmECF—RibU transporter was expressed in *E. coli* BL21(DE3) by autoinduction in ZYP-5052 media [62] at 25 °C. After harvesting, cells were resuspended in Buffer A (50 mM Tris, pH 7.5, 500 mM NaCl, 20% glycerol, and 10 mM imidazole) and lysed by cell disruption in an Avestin homogenizer. The membrane fraction was isolated by ultracentrifugation. Membranes were resuspended in 10 ml of Buffer A per gram of membrane and solubilized in 1% dodecyl-maltoside (DDM) for 1 hr at 4 °C with stirring. The LmECF—RibU transporter was bound to TALON resin (Clontech) in DDM and exchanged into SEC buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, and 0.06% undecyl-maltoside) by thrombin cleavage from washed beads. After removal from the Talon resin, all proteins were treated with 10 mM DTT and 10 mM MgCl₂, and further purified by gel filtration on a Superdex 200 column equilibrated in SEC buffer. Peak fractions were pooled, concentrated in Amicon 100K filtration devices, and were used immediately for assays or flash frozen and stored at –80 °C.

Riboflavin binding assays

The LmECF-RibU transporter with His199Ala and His206Ala of EcfA' and EcfA, respectively, was incubated with 2mM MgATP and various concentrations of riboflavin, as described in [37]. Subsequently, 50 µl of each sample was injected on a Shodex KW804 analytical SEC column (Thomson, Clear Brook, VA) equilibrated with 50 mM Tris, pH 7.5, 200 mM Na₂SO₄, 3 mM NaN₃ and 0.05 % DDM on an HPLC (Waters, Milford, MA) equipped with UV and fluorescence detectors. Riboflavin bound LmRibU was detected by monitoring the intrinsic riboflavin fluorescence ($\lambda_{\text{ex}} = 440 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$). FSEC peak positions and heights were determined with the Empower software (Waters). The identical protocol was used to measure riboflavin binding by the Tyr136Phe and Tyr136Gly mutants of RibU.

Homology modeling of LIRibU and LmRibU

The riboflavin binding EcfS subunits from *L. lactis* (LIRibU) and *L. monocytogenes* (LmRibU) share 26 and 33% identity with TmRibU, respectively. A homology model of each protein was constructed using Modeler v9.16 (<http://salilab.org/modeller/>) based upon the alignment shown in Fig. S2. The final models were energy minimized in PHENIX [60] prior to analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ECF	energy coupling factor
ATP	adenosine triphosphate
SEC	size exclusion chromatography
MALLS	multi-angle laser light scattering
TM	transmembrane

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Highlights

- The conformational changes upon substrate binding to EcfS proteins are unknown.
- Riboflavin-bound TmRibU is exceptionally stable compared to other membrane proteins.
- Seven of eight available riboflavin H-bonds are satisfied upon binding to TmRibU.
- A conserved aromatic residue in TM5, Tyr130, caps the substrate binding site.
- This aromatic capping interaction is essential for high affinity substrate binding.

Figure 1. Characterization of the riboflavin binding S subunit from *T. maritima*, TmRibU
 (a) Preparative SEC trace and SDS-PAGE of purified TmRibU visualized by Coomassie staining and UV fluorescence (inset). (b) Visible absorbance spectra of free riboflavin (gray) and purified TmRibU (black). (c) Pull down experiment in which His-tagged wild type TmRibU or a truncation lacking the final 10 amino acids (RibU Δ Ct) were co-expressed with the EcfA, A', and T subunits from *T. maritima* in *E.coli*. Both variants of TmRibU co-purified with the other components of the ECF module. (d) SEC-MALLS analysis of the oligomeric state of TmRibU in solution.

Figure 2. The crystal structure of riboflavin-bound TmRibU

(a) Ribbon diagram of TmRibU viewed parallel to the membrane with riboflavin shown in sphere representation. (b) Surface electrostatic potential of the riboflavin binding pocket viewed from the extracellular side. (c) and (d) Two views of the hydrophobic flavin binding cavity with relevant residues labeled. (e) The riboflavin binding site in TmRibU with H-bonds indicated as black dashed lines and side chains labeled. Loop1-2 is not shown for clarity. (f) Contact diagram of TmRibU residues that H-bond with the riboflavin transport substrate.

Figure 3. An aromatic lid encloses the riboflavin binding site

(a) Extracellular view of the substrate binding site in the TmRibU (top) and SaRibU (bottom) structures. Relevant side chains and bound riboflavin are shown as spheres. The disordered region in SaRibU is represented as dashed lines. (c) Sequence alignment of TM5 from RibU homologs. (d) Thermal melting curves of riboflavin-bound TmRibU and several mutants. The data are derived from two independent replicates for each sample.

Figure 4. Tyr130 of the PLY motif is required for high affinity substrate binding

Fluorescence size exclusion chromatography (FSEC) traces measured against riboflavin concentration for the WT (a), Tyr136Phe (b), and (c) Tyr136Gly mutants of LmRibU. (d) Riboflavin binding isotherms for each protein derived from the FSEC data. The data in (d) represent the mean and standard deviation of three independent replicates for each sample.

Figure 5. Structural conservation of the aromatic lid in other S subunits

(a) Ribbon diagram of the TmRibU structure. The conserved aromatic residue and bound substrate are shown in stick representation. (b) The thiamine-bound structure of ThiT. (c) Superposition of the TmRibU (pink) and ThiT (green) structures. The conserved aromatic residue in TM5 of FoIT (d), HmpT (e) and PanT (f), respectively.

Table 1

Data collection and refinement statistics

Data Collection			
Protein	TmRibU	TmRibU	TmRibU
PDB code	5KBW	5KC0	5KC4
Synchrotron	ALS	NSLS	NSLS
Beamline	8.2.1	X29	X25
Space group	P2 ₁	P6 ₅ 22	P2 ₁ 2 ₁ 2 ₁
Cell dimensions (Å)	40.1, 94.4, 50.9	72.6, 72.6, 232.9	65.8, 80.8, 118
Cell angles (°)	90, 90.8, 90	90, 90, 120	90, 90, 90
Resolution (Å)	40.0 -2.60 (2.80-2.60)	37.4 -3.2 (3.28-3.20)	40.0 - 3.4 (3.48-3.40)
R _{sym}	0.080 (0.38)	0.058 (0.62)	0.078 (0.89)
I/σI	19.8 (2.9)	31.3 (3.3)	24.6 (3.1)
Completeness (%)	97.3 (75.3)	91.2 (83.9)	94.5 (87.7)
Redundancy	2.8 (2.4)	20.9 (18.8)	14.1 (14.3)
Refinement			
No. reflections	10106	6007	8513
R _{work} /R _{free}	0.211/0.255	0.258/0.286	0.249/0.269
No. atoms			
Protein	2643	1277	2542
Ligand	54	27	54
Solvent	7	42	42
B factors (Å ²)			
Protein	54.0	87.6	101.4
Ligand	52.5	73.4	113.2
Solvent	53.7	116.7	139.3
R.m.s. deviations			
Bond lengths (Å)	0.004	0.006	0.004
Bond angles (°)	0.92	0.95	0.90
Ramachandran plot			
Most favored (%)	94.1	92.1	90.1
Allowed (%)	5.9	6.1	7.1
Outlier (%)	0.0	1.8	2.8
MolProbity score	1.64	1.95	2.17

Notes:

$R_{\text{merge}} = \frac{\sum_{\text{hkl}} \sum_i |I_i^{\text{hkl}} - \langle I^{\text{hkl}} \rangle|}{\sum_{\text{hkl}} \sum_i I_i^{\text{hkl}}}$, where I is the intensity of a reflection hkl and $\langle I \rangle$ is the average over measurements of hkl.

Redundancy represents the ratio between the number of measurements and the number of unique reflections.

R factor = $\sum |F_{\text{obs}} - F_{\text{cal}}| / \sum F_{\text{obs}}$; 5% of the data that were excluded from the refinement were used to calculate R_{free} . The average B factor was calculated for all non-hydrogen atoms.

r.m.s.d. is the root-mean-square deviation of the bond angle and length.

Numbers in parentheses are statistics of the highest resolution shell.

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